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REDUCED SURVIVAL OF ADULT *CULEX PIPIENS* INFECTED WITH RIFT VALLEY FEVER VIRUS

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Abstract. The effect of Rift Valley fever (RVF) viral infection on the survival of female *Culex pipiens* was examined. In 3 experiments in which mosquitoes ingested RVF virus, there was a 44% decrease in survival to days 14–16 for transmitting vs. nontransmitting mosquitoes, and a 48% decrease in survival for individuals with disseminated vs. nondisseminated infections. These results were corroborated by other experiments in which survival of mosquitoes intrathoracically inoculated with RVF virus was compared with that of those inoculated with diluent. In both the per os and inoculation tests, uninfected mosquitoes survived significantly longer than infected mosquitoes. Even though mosquitoes with disseminated infections had a lower survival rate than did uninfected mosquitoes, dissemination and transmission rates were similar at days 7 and 14–18 after the infectious bloodmeal. This suggests that nondisseminated individuals were developing disseminated infections and becoming capable of transmitting virus between days 7 and 14–18 at approximately the same rate older transmitters were dying. The decreased survival associated with RVF viral infection should be considered in predictive models of this disease.

Traditionally, arboviral infections are not believed to harm their invertebrate vector.[1] This view has been challenged by several recent studies. Mims et al.[2] found that Semliki Forest viral infection resulted in degeneration of the salivary glands in *Aedes aegypti*. In addition, morphological changes in the salivary glands, such as proliferation and clumping of smooth endoplasmic reticulum, extensive vesicle formation, and distention of the rough endoplasmic reticulum, have been reported for mosquitoes infected with various arboviruses.[3–6] Arboviruses also have been shown to have detrimental physiological effects on mosquitoes. A reduced ability to refeed has been described in *Ae. triseriatus* orally infected with La Crosse virus[7] and in *Culex pipiens* infected with Rift Valley fever (RVF) virus.[8] Decreased fecundity has been reported in *Cx. pipiens* infected with RVF virus[8] and, possibly, in *Ae. albopictus* infected with either San Angelo or Kunjin viruses.[9] Several investigations have shown that transovarially infected larvae require more time to develop to the pupal stage than their uninfected siblings.[9–11] In contrast, no significant effect of viral infection on duration of the larval stage or on refeeding ability was found in *Ae. triseriatus* transovarially infected with La Crosse virus,[12, 13] or on adult survivorship in *Cx. pipiens* infected with Japanese B encephalitis virus[14] or in *Ae. aegypti* infected with Semliki Forest virus.[2] We examined the possible effect of RVF viral infection on survival of adult females of *Cx. pipiens*. This mosquito, a species not ordinarily associated with RVF virus, was implicated as the primary vector during the major epizootic of RVF that occurred in Egypt from 1977–79.[15]

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MATERIALS AND METHODS

Mosquitoes, virus, and viral assay procedures

Four- to 8-day-old female *Cx. pipiens* from the F_{51} to F_{53} generations of the El Gabal strain,[16]

derived from specimens collected in Egypt in 1981, were used in this study. Immatures and adults were reared in incubators maintained at 26°C (±1°C) with a 16L:8D hr photoperiod. Larvae were fed a mixture of liver powder, yeast, and ground hog chow. High relative humidity was maintained for adults by placing moist gauze pads on top of the containers in which the mosquitoes were kept.

The ZH-501 strain[17] of RVF virus was used in each experiment. This strain was isolated in 1977 from a human case in Egypt and passed twice in fetal rhesus lung cells. Mosquito bodies and legs were triturated separately in 1 ml of mosquito diluent (10% calf serum in Medium 199 with Hanks' salts plus antibiotics) and then stored at −70°C until assayed for virus by plaque assay on 2–4-day-old Vero cell monolayers.[16] All viral titers represent plaque forming units (pfu) per specimen.

Survival of mosquitoes orally exposed to RVF virus

In 3 experiments, mosquitoes were exposed to RVF virus by allowing them to feed on an anesthetized viremic hamster that had been infected by intraperitoneal inoculation with 10^4 pfu of RVF virus in 0.2 ml of diluent about 24 hr prior to mosquito feeding. Engorged mosquitoes were placed in a 3.8 l cardboard cage. Three days later, a dish containing 50 ml of water was added to each cage to stimulate oviposition. Seven to 9 days after the infectious bloodmeal, mosquitoes were individually exposed for about 1 hr to uninfected, restrained hamsters[16] to test for their ability to transmit virus. In all experiments, the right mesothoracic leg was removed immediately following feeding and assayed for virus to determine if virus had disseminated from the midgut.[18] The mosquitoes were then held individually in 0.9 l cardboard containers with screened lids. To facilitate oviposition, a cup containing 15 ml of water was added to each container on the third day post-engorgement. In experiments 2 and 3, dead mosquitoes were removed daily and the date of death recorded. In each experiment, a second transmission test was made between days 14 and 18 post-viremic bloodmeal. Following this test, the mosquitoes' bodies and remaining legs were triturated separately in 1 ml of diluent and held at −70°C until assayed.

Survival of mosquitoes inoculated with RVF virus

Experiments 4 and 5 were conducted with inoculated mosquitoes to eliminate the possibility that mosquitoes genetically or environmentally (i.e., larval nutrition) more susceptible to infection by oral exposure were also less hardy, and to increase the sample size of mosquitoes with a disseminated viral infection. In these experiments, mosquitoes were inoculated[19] with either diluent or 10^2 pfu of RVF virus (10^3 *Cx. pipiens* ID_{50}).[18]

Experiment 4 was conducted blind so that the person inoculating the mosquitoes did not know if the inoculum contained diluent or virus. All of these mosquitoes were then placed in a single 3.8 l cardboard container with netting at one end. Mortality within 48 hr was considered to have resulted from trauma due to the inoculation procedure and these individuals were discarded. Mosquitoes that died after 48 hr were assayed individually for virus. Previous studies have shown that RVF virus is still detectable in *Cx. pipiens* for at least 48 hr after mosquito death (M. E. Faran and M. J. Turell, personal communication). Because mosquitoes were checked daily, recovery of virus from a dead mosquito indicated that it was infected (virus-inoculated), while failure to recover virus from an inoculated mosquito indicated that it was uninfected (diluent-inoculated).

In experiment 5, the RVF virus- and diluent-inoculated mosquitoes were placed in separate cages, but otherwise treated the same as in experiment 4. Dead mosquitoes were removed and counted daily. *Cx. pipiens* infected with RVF virus by inoculation are capable of transmitting virus by day 2 or 3.[20] Therefore, it was not necessary to conduct transmission attempts for these experiments.

Statistical analysis

Comparisons betwen survival rates were performed by using Fisher's exact test (FET)[21] at the 0.05 significance level. Survival curves were compared by using methods of survival analysis to compare survival profiles over the duration of the experiment. The Kaplan-Meier product limit estimator (PL)[22] of the survival curves was used to estimate median time to death. The Man-

TABLE 1

Seven-day survival rates following first viral transmission tests for Culex pipiens *orally exposed to Rift Valley fever virus*[a]

	Experiment			Total
	1	2	3	
Overall survival	43/83 (52)†	45/93 (48)	24/58 (41)	112/234 (48)
Survival:				
Nontransmitters	29/41 (71)	37/72 (51)	20/39 (51)	86/152 (57)
Transmitters	14/42 (33)	8/21 (38)	4/19 (21)	26/82 (32)
Nondisseminated	28/36 (78)	37/69 (54)	16/29 (55)	81/134 (60)
Disseminated	15/47 (32)	8/24 (33)	8/29 (28)	31/100 (31)

[a] First transmission test on days 8–9 (exp. 1) or days 7–8 (exps. 2, 3) following the infectious bloodmeal.
† Number alive 7 days after the first transmission test/No. at the first transmission test (% survival).

tel-Cox (MC) *P* value[22] tests whether the survival curves have the same profiles, utilizing information on all mosquitoes entering the study to estimate mortality profiles.

RESULTS

Survival of mosquitoes orally exposed to RVF virus

In experiments 1–3, mosquitoes ingested between $10^{5.6}$ and $10^{6.9}$ pfu of RVF virus from the viremic hamsters. Percent survival was based on the number of mosquitoes in each group living for at least 7 days after the first transmission test. Overall survival rates for the 3 experiments were similar and ranged from 41% to 52% (Table 1). Nontransmitters consistently survived at a higher rate than did transmitting mosquitoes (86/152 [57%] vs. 26/82 [32%]). By using FET, the differences between transmitters and nontransmitters were significant in experiments 1 and 3, but not significant in experiment 2 (Table 2).

Survival rates for individuals with nondisseminated infections were significantly higher than those for individuals with disseminated infections in experiments 1 ($P < 0.001$) and 3 ($P = 0.031$) (Table 2). This difference was borderline ($P = 0.069$) in experiment 2.

Because daily survival rates were recorded for all experiments except the first, we were able to compare the survival profiles for experiments 2–5 by using the PL estimator and MC *P* values (Table 2). For experiments 2 and 3, comparisons were made between the transmitters and nontransmitters, and the disseminated and nondisseminated infection groups. While differences were not significant in experiment 2, the differ-

TABLE 2

Statistical analysis of survival data for Culex pipiens *orally exposed to Rift Valley fever virus*

	Experiment			Total
	1	2	3	
Fisher's exact test				
Disseminated vs. nondisseminated	<0.001[a]	0.069	0.031	<0.001
Transmitters vs. nontransmitters	<0.001	0.205	0.026	<0.001
Mantel-Cox *P* value				
Disseminated vs. nondisseminated	—	0.154	0.038	0.004
Transmitters vs. nontransmitters	—	0.366	0.045	0.021
Mean (median) time to death (days)				
Nondisseminated	—	6.0 (>8)	5.7 (>8)	5.9 (>8)
Disseminated	—	5.5 (5.5)	4.3 (3.0)	4.8 (5.0)
Nontransmitters	—	6.0 (>8)	5.4 (>8)	5.8 (>8)
Transmitters	—	5.5 (6.0)	4.1 (3.0)	4.9 (5.0)

[a] *P* value.

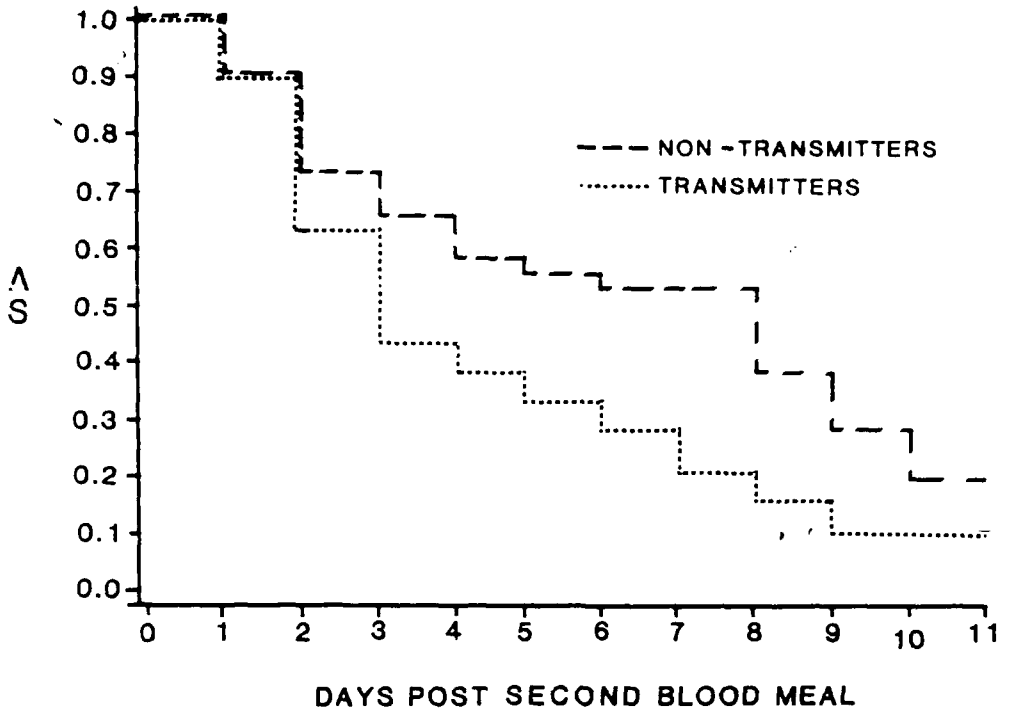

FIGURE 1. Kaplan-Meier product-limit estimate for probability of survival from one day to the next for RVF virus-transmitting vs. nontransmitting *Cx. pipiens* in experiment 3.

ences between transmitters vs. nontransmitters and between disseminated vs. nondisseminated mosquitoes in experiment 3 were significant (Table 2). Likewise, the MC P values were <0.05 for both sets of comparisons in the combined data for experiments 2 and 3. The survival curves generated by the PL estimator for transmitters and nontransmitters in experiment 3 are shown in Figure 1.

Transmission rates at the second transmission test were 20% higher than at the first test, but this difference was not significant (Table 3). Viral dissemination rates were similar at the first and second transmission tests. However, 18/45 (40%) of the mosquitoes with disseminated infections at the second transmission test developed these infections after the first transmission test. Similarly, 11/26 (42%) of the mosquitoes transmitting virus at the second transmission test had not transmitted virus during the first transmission test. Two of the 18 individuals that had disseminated infections, but did not transmit virus at the first test, survived to the second test. Both of these mosquitoes transmitted virus at this test. During the second transmission test, 6/32 (19%) of the mosquitoes with disseminated infections engorged but did not transmit virus. None of these 6 mosquitoes had a disseminated infection at the first test. While the mean amount of virus in the mesothoracic leg of transmitters was greater than that of disseminated, nontransmitters at the first transmission attempt in all 3 experiments, this difference in titers was significant ($P < 0.001$, t-test) only in experiment 3 (Table 4). Similarly, in the second transmission test, while viral titers of the legs of transmitters were generally greater than those of nontransmitters, these differences were not significant. Comparison of refeeding rates between individuals with and without disseminated infections revealed no statistically significant differences in all 3 experiments (data not shown).

Survival of mosquitoes inoculated with RVF virus

The survivability of female mosquitoes inoculated with virus or diluent was compared by using the PL estimator and MC P values (Table

TABLE 3

Transmission and dissemination rates of Culex pipiens *orally exposed to Rift Valley fever virus following viral transmission test*

	Experiment			Total
	1	2	3	
Transmission rate @ 1st TT*	42/83 (51)†	21/93 (23)	19/58 (33)	82/234 (35)
Transmission rate @ 2nd TT	16/30 (53)	6/22 (27)	4/10 (40)	26/62 (42)
New transmitters @ 2nd TT/ transmitters @ 2nd TT	6/16 (38)	3/6 (50)	2/4 (50)	11/26 (42)
Dissemination rate @ 1st TT	47/83 (57)‡	24/93 (26)	29/58 (50)	100/234 (43)
Dissemination rate @ 2nd TT	29/43 (65)	12/44 (27)	5/10 (50)	46/97 (47)
New disseminated @ 2nd TT/ disseminated @ 2nd TT	14/28 (50)	4/12 (33)	0/5 (0)	18/45 (40)

* TT = transmission test. First transmission test on days 8, 9 (exp. 1) and days 7, 8 (exps. 2, 3). Second transmission test 7 days after the first transmission test (exps. 1, 2) and 9–10 days after the first transmission test (exp. 3).

† Number transmitting/No. feeding (% transmitting).

‡ Number with virus in their legs/No. tested (% with virus in their legs).

5). In experiment 4, the mean times to death for the uninfected and the infected groups were 15 and 12 days, respectively. Likewise, in experiment 5, the uninfected mosquitoes survived a mean of 30 days as compared to a mean of 22 days for the infected mosquitoes. These differences in survival between the uninfected and infected groups are highly significant ($P = 0.03$ and $P < 0.001$) in experiments 4 and 5, respectively, and are graphically represented for experiment 5 (Fig. 2). Mean times to death were also greater for the uninfected mosquitoes than for the infected mosquitoes at the 25th and 75th quartiles in both experiments.

DISCUSSION

We believe this is the first report of an arbovirus decreasing adult survival of an epizootic vector mosquito. However, while *Cx. pipiens* was implicated in the Egyptian epizootic, it does not appear to be involved in the enzootic cycle of RVF virus. Therefore, it is possible that the effects we have observed would not occur in an enzootic maintenance species that has coevolved with RVF virus. Also of potential importance in the interpretation of our results is the possibility that genetic changes have occurred during the establishment and maintenance of our colony.

The PL estimate provides a more accurate comparison of survival data between the different groups than does FET, because the former allows comparison of the survival profiles over the duration of the experiment, whereas, the latter allows comparison of survival values only at the end of an experiment. The potential problem in comparing endpoints is that curves may converge toward the end of an experiment, but may have been very different earlier.

In experiments 1–3, 18/100 (18%) of the mosquitoes with a disseminated infection at their first transmission test and 6/32 (19%) of those at the second test did not transmit virus. This failure to transmit virus was probably due to an insufficient time interval between viral dissemination and the transmission attempt rather than to the action of a salivary gland infection barrier.[23] This is supported by studies in which all *Cx. pipiens* intrathoracically inoculated with RVF virus transmitted this virus by bite.[20] However,

TABLE 4

Titer of Rift Valley fever virus in legs of Culex pipiens *after first and second transmission attempts*

Experiment	1st transmission attempt		2nd transmission attempt	
	Nontransmitters	Transmitters	Nontransmitters	Transmitters
1	3.2 (2.5–3.6)*	3.5 (2.2–4.0)	3.9 (3.5–4.2)	4.0 (3.6–4.3)
2	2.9 (2.5–3.2)	3.0 (2.8–3.4)	3.7	3.6 (3.3–3.8)
3	2.9 (2.0–3.6)	3.9 (2.7–4.6)	3.5	4.1 (3.8–4.4)

* Mean (range) viral titers expressed as $\log_{10}$ pfu per leg.

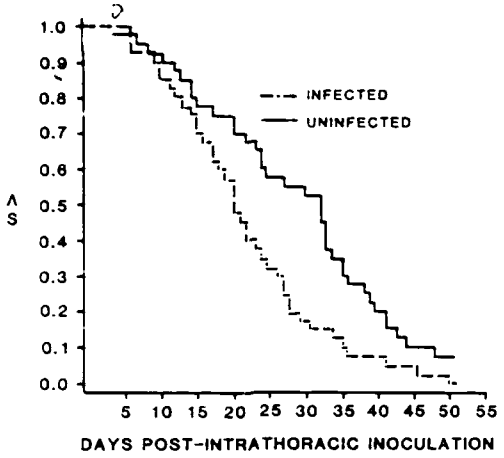

FIGURE 2. Kaplan-Meier product-limit estimate for probability of survival from one day to the next for uninfected (diluent-inoculated) and infected (RVF virus-inoculated) *Cx. pipiens* in experiment 5.

in those studies there was a delay of 1–2 days after entry of virus into the hemocoel (inoculation) before the mosquitoes were able to transmit virus. Also, both mosquitoes in the present study with a disseminated infection that failed to transmit virus at the first test and survived to the second test, transmitted virus at that test. In addition, the generally lower titers in the legs of nontransmitters, as compared to the titers in the legs of transmitters, may represent recent dissemination of virus from the midgut.

The differences between the survival of infected (virus-inoculated) and uninfected (diluent-inoculated) mosquitoes were highly significant. While there was about a 25% reduction in the mean time to death for the virus-inoculated mosquitoes as compared to the diluent-inoculated mosquitoes in both experiments 4 and 5, the individuals in experiment 5 survived an average of about 15 days longer than those in 4. All the virus-inoculated mosquitoes in these experiments were potential transmitters,[20] so that the comparison can be viewed as transmitters vs. uninfected or as disseminated vs. uninfected. The use of inoculated specimens supports the finding that orally exposed *Cx. pipiens* that are capable of transmitting RVF virus or have developed a disseminated RVF viral infection have reduced survival as compared to those individuals that failed to transmit virus or to develop a disseminated infection. Also, because the mosquitoes

TABLE 5

Statistical analysis of survival data for Culex pipiens *intrathoracically inoculated with either Rift Valley fever virus or diluent*

	Experiment	
	4	5
Mantel-Cox *P* value		
Infected vs. uninfected	0.003	0.001
Mean (median) time to death (days)		
Uninfected	15.0 (15.0)	29.7 (32.0)
Infected	11.9 (12.0)	21.8 (20.0)

were inoculated with virus or diluent at random, it is unlikely that the reduced survival was due to genetically less hardy individuals being more susceptible to RVF viral infection or dissemination.

We also found that 11/26 (42%) of the mosquitoes that transmitted virus to hamsters during the second transmission test did not do so at the first test. In addition, 18/45 (40%) of the mosquitoes with a disseminated infection at the second transmission test did not have detectable virus in their legs at the first transmission test. Thus, dissemination of virus to the hemocoel and development of the ability to transmit RVF virus occurred between days 7 and 15 after the infectious bloodmeal. This may explain why even though the survival rate of transmitters was much less than that of nontransmitters, the transmission rate at the second transmission test on days 15 and 16 was almost the same as at the first transmission test 7 days earlier. Another explanation for the similarity of transmission rates at the first and second transmission tests could be increased refeeding by infected mosquitoes. However, we found no significant differences in refeeding rates in the small samples in this study, and Turell et al.[8] reported a 21% reduction in refeeding rates for *Cx. pipiens* with a disseminated RVF viral infection. It appears, therefore, that mosquitoes incapable of transmitting virus were becoming competent vectors at a rate which compensated for the excess mortality observed for the previous transmitters as compared to the nontransmitters. Escape of virus from the alimentary canal may be sporadic in the population, or triggered by some event, such as refeeding or oviposition.

The phenomenon of reduced survival ob-

served for *Cx. pipiens* infected with RVF virus should be examined in long-established, enzootic, vector-virus systems. If our findings are corroborated, reduced survival of infected vectors and continued addition of new transmitters should be incorporated into predictive models.

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